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Studies of Rhodamine-123: Effect on Rat Prostate Cancer and Human Prostate Cancer Cells In Vitro

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The effect of the lipophilic, cationic dye, Rhodamine-123 (Rh-123), on prostate cancer in rats, and on three tumor cell lines in vitro is reported here. The general toxicity of Rh-123 in mice has been found to be minimal. Lobund-Wistar (L-W) rats with the autochthonous prostate cancer of Pollard were treated for six doses with Rh-123 at a dose of 15 mg/kg subcutaneously every other day. Microscopic examination of the tumors revealed cellular and acinar destruction. The effectiveness of Rh-123 as a cytotoxic agent was tested by clonogenic and viability assays in vitro with three human prostate cancer cell lines. Severe (60–95%) growth inhibition was observed following Rh-123 exposure for 2–5 days at doses as low as 1.6 μg/ml in all three prostate cancer cell lines.

KEY WORDS: **mitochondrial toxin, autochthonous rat prostate tumor, chemotherapy, prostate cancer, Rhodamine-123**

INTRODUCTION

Metastatic hormone refractory prostate cancer has responded poorly to chemotherapy because of its slow rate of replication [1]. New agents for treatment of this disease are needed that exert their effect independent of the rate of cell division or of their ability to interfere with DNA or RNA metabolism. We propose Rhodamine-123 (Rh-123) as one such agent. Rh-123, localizing in the mitochondria of living cells [2], is selectively toxic for carcinoma cells because of a difference in the plasma membrane potential of normal and malignant cells together with the positive charge on this lipophilic molecule [3]. In 1986, Arcadi [4] reported the effect of this agent on the transplantable rat prostate tumor R3327-H (Dunning). Rh-123 was administered subcutaneously every other day at a dosage of 15 mg/kg body weight for 52 days. There was significant destructive alteration of the acinar cells with disruption of the cells from the basement membrane, destruction of the cytoplasm, as well as vacuolization and change in fibroblast shape and density.

The highly malignant, androgen-independent transplantable tumor designated P-A III, was reported in 1990 to be highly sensitive to Rh-123 [5]. Rh-123 treatment of the tumor resulted in significant destruction of tumor cells, with no toxicity noted in normal cells. Injection of tumor remnants into untreated susceptible Lobund-Wistar (L-W) rats produced no tumor growth. An additional rat prostate study is presented in this paper which examines the effect of Rh-123 on the autochthonous rat prostate adenocarcinoma produced in L-W rats by the injection of N-methyl-N-nitrosourea (MNU) and testosterone propionate (TP) [6].

In Arcadi's initial studies [4,5] on rats dimethylsulfoxide (DMSO) was used as a solvent for Rh-123. Since DMSO was not satisfactory for intravenous use in humans, an alcohol-glucose solution was devised. This paper includes a study of the toxicity of these two solvents and Rh-123 in mice. Further studies presented in this paper are those relating to the effect of Rh-123 on various human prostate cancer cell lines. These include the effect of Rh-123 on clonogenicity (plating efficiency), Rh-123 uptake and retention, and Rh-123-induced cytotoxicity.

MATERIALS AND METHODS

Rh-123, laser grade, $C_{21}H_{17}ClN_2O_3$ with a molecular weight of 380.83 was purchased from the Eastman Kodak Company (Rochester, NY).

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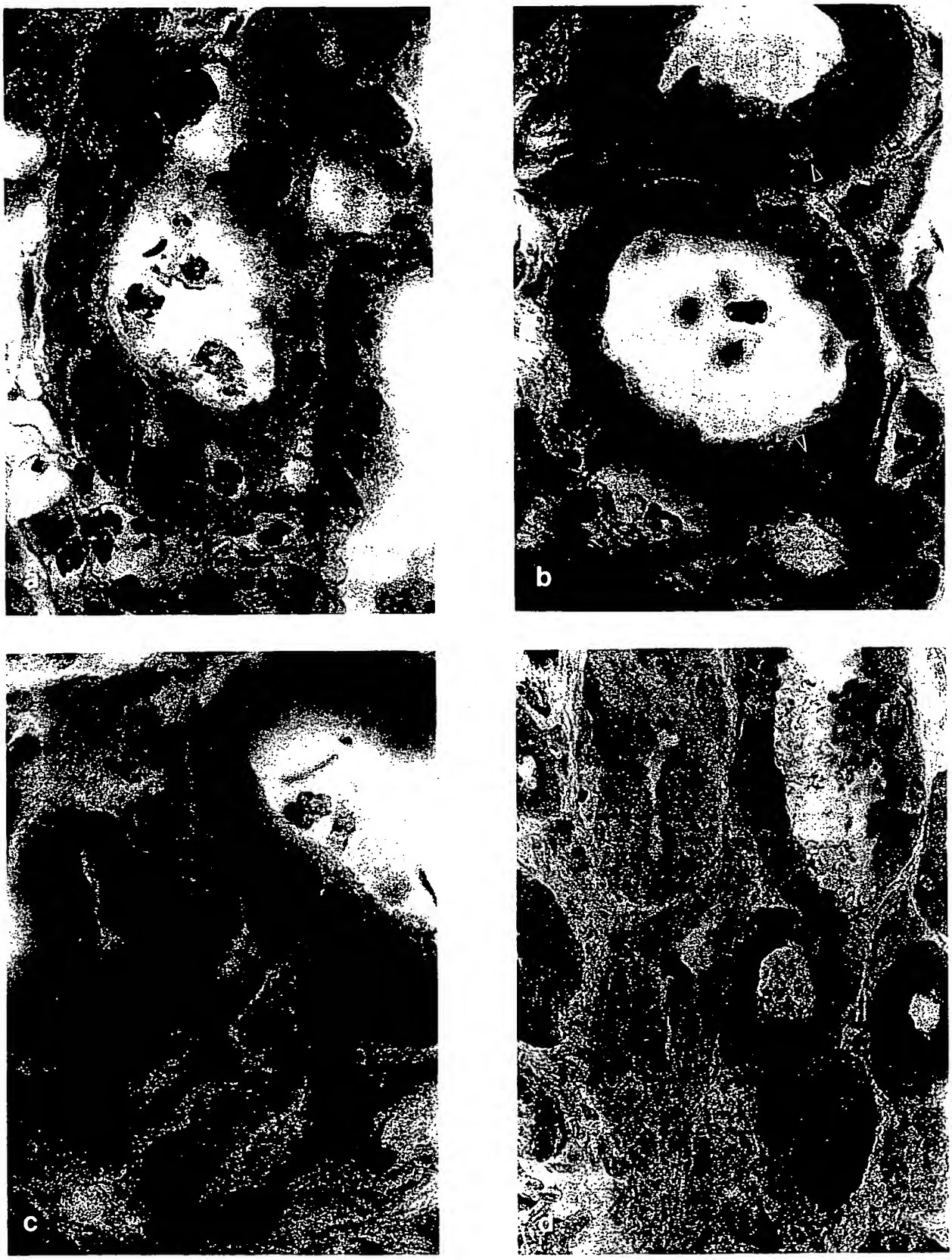

Fig. 1 (legend on following page).

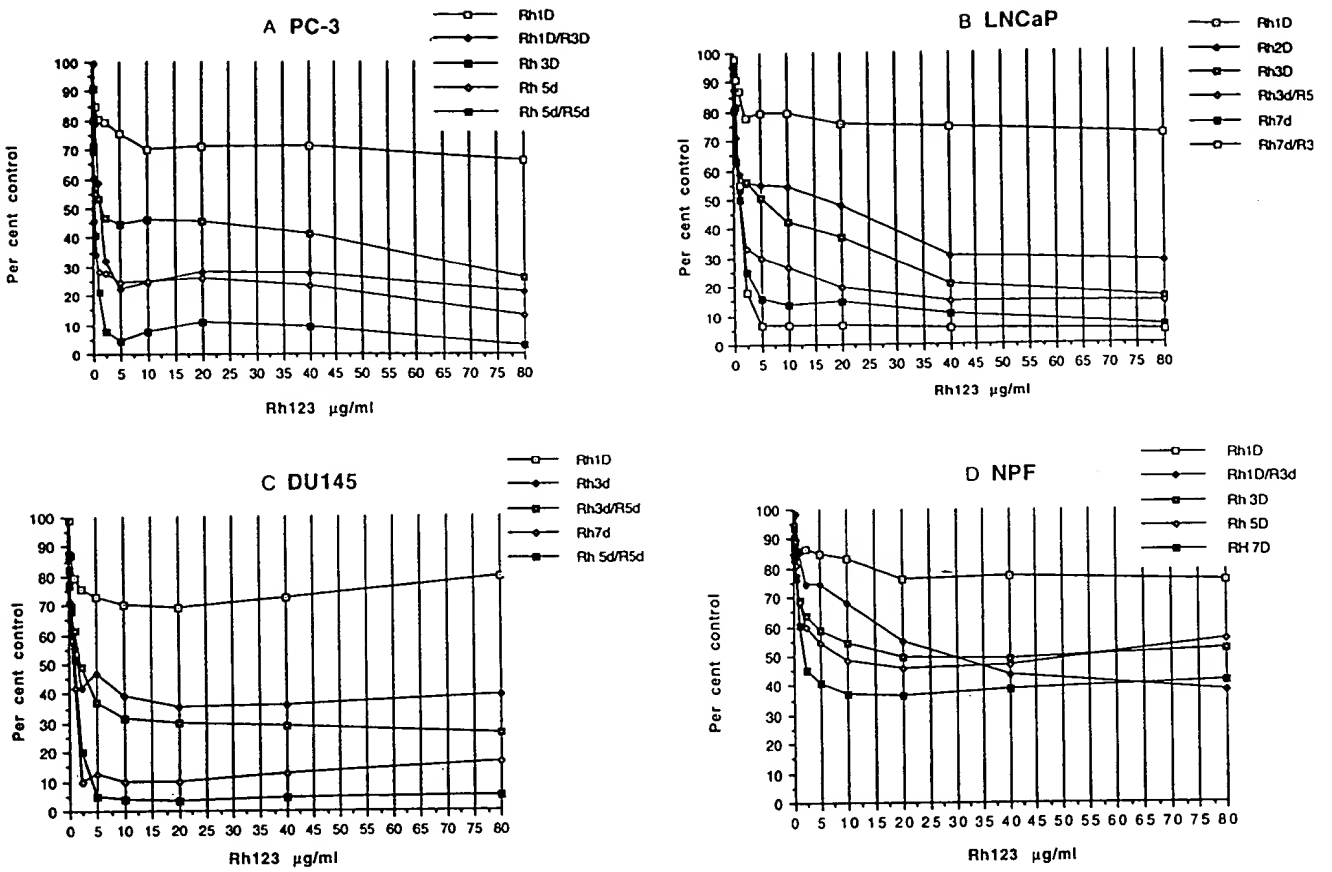

Fig. 2. **A:** Effect of Rh-123 exposure over a 0–80 µg/ml concentration range in PC-3 prostate cancer cells. Data shown as per cent viable cells (expressed relative to the viable cells in control, untreated sister wells) present following exposure to different Rh-123 concentrations for 1, 2, 3, 5, or 7 days. Values shown for each Rh-123 concentration are averages of 8 or 16 duplicate wells. Viability of cells were determined as described in Materials and Methods. *Abbreviations:* Rh1d, Rh2d, Rh3d, Rh7d—cells exposed to Rh-123 continuously for 1, 2, 3 or 7 days. *Rh 1d/R3D*—cells exposed to Rh-123 for 1 day plus recovery in normal growth medium for 3 days before cell viability assessment. *Rh 3d/R5*—Rh-123 exposure 3 days plus recovery for 5 days-cell viability assessment. *Rh7d/R3*—Rh-123 exposure 7 days plus 3 days recovery period in normal growth medium before cell viability assessment. **B:** Effect of Rh-123 exposure over a 0–80 µg/ml concentration range in LNCaP prostate cancer cells. Data from same experiment as for A. See A for other details and abbreviations. **C:** Effect of Rh-123 exposure over a 0–80 µg/ml concentration range in DU145 prostate cancer cells. Data from same experiment as for A. See A for other details and abbreviations. **D:** Effect of Rh-123 exposure over a 0–80 µg/ml concentration range in NPF non-tumorigenic, diploid prostate cells. *Note*: growth inhibition due to Rh-123 treatment is much less in comparison to that observed with tumorigenic cells (A, B, C). Data from same experiment as for A. See A for other details and abbreviations.

Rat Prostate Adenocarcinoma

This study was designed to determine the effectiveness of Rh-123 on induced autochthonous rat prostate adenocarcinoma that developed within the prostate gland and seminal vesicles of L-W rats. Thirteen L-W rats were inoculated intravenously with acidified MNU (30 mg/kg body weight [BW]). Following the single inoculation of MNU, the rats were implanted subcutaneously with TP (50 mg) sealed in a silastic tube. Three implants of TP were administered, each at intervals of 2 months. After a latent period of 4–6 months, small palpable tumors were detected in the abdomen. They were then administered Rh-123 (15 mg/kg BW) subcutaneously every other day for six doses. The Rh-123 was dissolved in a 5% ethanol-5% glucose solution at a concentration of 5 mg/ml. The rats were sacrificed 1 week after the last dose of Rh-123 and their tissues were fixed in 10% formalin.

Fig. 1. **a:** Untreated autochthonous rat prostate complex adenocarcinoma (ARP CA). Note irregular nuclei with prominent nucleoli; cytoplasm is plentiful and well-defined. **b:** ARPCA treated with Rh-123, 15 mg/kg BW every other day for six doses. Cytoplasm is greatly decreased in volume and the nuclei are smaller and less distinct. Cyst formation is noted in both acini (arrows). **c:** ARPCA treated as in b. Large cyst of cytoplasm between two nuclei is shown. **d:** ARPCA treated as in b. Note smudging and loss staining of cytoplasm. Nuclear detail is lost. Hemotoxylin and eosin stained sections. Original magnification ×400.

Toxicity Studies

The toxicity of two solvents for Rh-123, DMSO and alcohol-glucose, was studied in 60-day-old Swiss-Webster mice (Simonsen Laboratories, Inc., Gilroy, CA). For

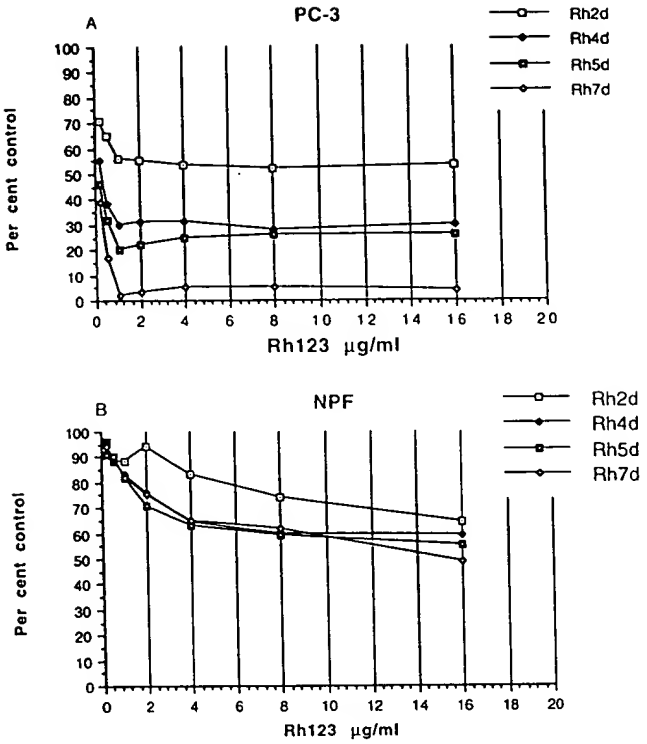

Fig. 3. **A:** Growth inhibition in PC-3 prostate cancer cells due to Rh-123 exposure at concentrations of 0–16 µg/ml for 1–7 days. This graph better illustrates the maximum cytotoxic effects noted at low Rh-123 concentrations. See Figure 2A for other details and abbreviations. Data shown are from a different experiment than that shown in Figures 2A–2D. **B:** Growth inhibition in NPF non-tumorigenic prostate fibroblasts due to Rh-123 exposure at concentrations of 0–16 µg/ml for 1–7 days. Compare with A. Data from same as experiment as for A. See Figure 2A for other details and abbreviations.

each solvent group there were six groups of five mice each with the dose per group 2.0 mg/kg, 7.5 mg/kg, and 20 mg/kg. The solvents were at a concentration of 50% for the DMSO, and 5% alcohol in 5% glucose, and the concentration of Rh-123 was 5 mg/ml. The appropriate controls were utilized. Mice were injected subcutaneously every other day for 2 weeks.

Studies In Vitro

Assays of Rh-123 toxicity were done with three human prostate cancer cell lines, PC-3 [7], DU 145 [8], and LNCaP [9], and a nontumorigenic diploid prostate fibroblast cell strain (NPF-209) derived in our laboratories from a normal adult prostate. The NPF cells were used as controls, for comparison. The cells were maintained in disposable plastic culture vessels in a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12, Sigma Chemical Co, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; HyClone Labs, Inc., Logan, UT). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), DMSO, and other chemicals were obtained from Sigma.

Rh-123 was dissolved in high purity water (Milli Q, Millipore Corp., Bedford, MA) at 2 mg/ml and sterilized by 0.2 µm filtration before use for cell cultures. Two different in vitro assays were utilized to assess cell viability following Rh-123 treatment: i) colony formation by a clonal assay procedure [10] and ii) viability of cells determined by the MTT assay utilizing previously described techniques [11].

Clonogenic Potential of Treated Cells

Clonogenic potential of treated cells was determined on 24-hour old cells seeded in 60 mm disposable dishes ($1–2 \times 10^2$ cells/dish). The cells were exposed to Rh-123 for 24, 48, or 72 hours in triplicate sets at final concentrations of 1–50 µg/ml. They were then washed, and reincubated with Rh-123-free culture medium for 10–14 days before fixation, staining, and counting of colonies consisting of eight or more cells. Data are reported relative to the number of colonies observed in control (untreated) cultures carried in parallel and represent results confirmed by repeat experiments.

Cytotoxicity Due to Rh-123

Cytotoxicity due to Rh-123 treatment was determined with cells grown in 96-well disposable microtiter plates. Cells were seeded at $2.5–4 \times 10^3$ cells per well and allowed to grow in normal culture medium for 2–3 days to obtain cells in exponential growth phase. The cells were then exposed to various Rh-123 concentrations (in sets of eight wells per concentration) spanning the range of 0–80 µg/ml by adding appropriate amounts of sterile Rh-123 stock solution to an initial row and serial dilutions in the subsequent rows of cells with an automatic dispensing device to obtain the desired range of concentrations for each experiment. Each plate had one row of cells not exposed to Rh-123 that served as control cultures. Cytotoxicity determinations were done daily over a period of 1–8 days of Rh-123 exposure. Two microtiter plates were taken for each time point tested, with one used for immediate viability assessment and the other for testing the ability of Rh-123-treated cells to recover and grow following termination of exposure. For this, medium from wells of the treated plates was completely removed and the wells washed with serum-free medium before incubation with fresh 10% FBS containing DMEM/F12 culture medium for a subsequent 2–5 days before subjecting to cell viability determinations.

Determination of Viability of Cells

Determination of viability of cells in the microtiter plates was done by incubation of the cells with 0.4 mg/ml MTT for 4 hours at 37°C, subsequent removal of the medium, and dissolving the cell bound dye in 150 µl DMSO. The plates were next read at A_{540} nm with an Emax precision microplate reader (Molecular Devices

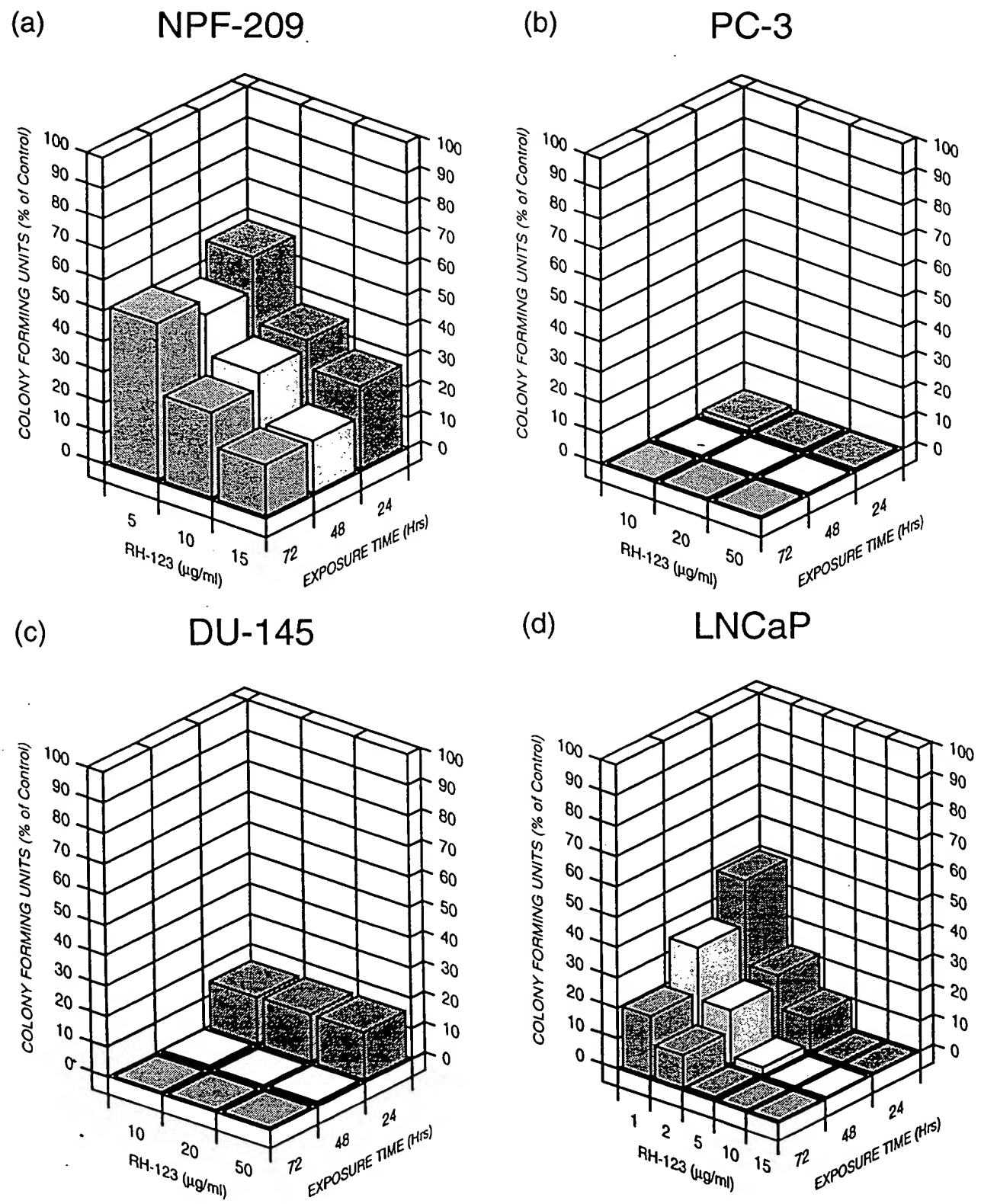

Fig. 4 a: The effect of Rhodamine 123 on colony growth in NPF non-tumorigenic prostate cells. Data shown as relative number of colonies following exposure to 5, 10, and 15 µg/ml Rh-123 for 24, 48, or 72 hrs. Number of colonies in treated dishes were expressed as percent of colonies detected in control, untreated sister cultures. See Materials and Methods for details of procedures used. b: The effect of Rhodamine 123 on colony growth in PC-3 prostate cancer cells. Other details are as in A. c: The effect of Rhodamine 123 on colony growth in DU145 prostate cancer cells. Other details are as in A. d: The effect of Rhodamine 123 on colony growth in LNCaP prostate cancer cells. Other details are as in a.

Corporation, Menlo Park, CA). MTT is reduced to an insoluble formazan by mitochondria in living cells. The cell bound dye is dissolved with DMSO and spectrophotometrically quantitated by absorbance at 540 nm. The OD_{540} nm reading is a measure of the number of viable cells present in the test sample. Growth inhibition due to Rh-123 treatment was determined relative to readings obtained with control (untreated) culture wells on each microtiter plate. Results were confirmed by two to three repeat experiments with each cell line.

Determination of Rh-123 Retention by Cells In Vitro

Rh-123 retention by the cells used in this study was determined by flow cytometry utilizing an EPICS Profile II Flow Cytometer (Coulter Corp., Miami, FL). Subconfluent cell cultures were exposed to Rh-123 for 1 hour, washed, and incubated for 24 hours in Rh-123-free culture medium. Rh-123 uptake and retention was determined by comparing fluorescence intensities (at an excitation wavelength of 488 nm) of 10,000 cells collected immediately after 1-hour Rh-123 exposure and 24 hours after termination of Rh-123 exposure.

RESULTS

Rat Prostate Adenocarcinoma

No gross changes were noted in the tumor mass of the prostate complex. Microscopic examination of the treated rats' prostate complexes revealed tumor tissue with marked cellular and acinar destruction, pyknosis, cytoplasmic smearing, and intraepithelial cyst formation (Fig. 1). The tumor mass had not decreased in size presumably because of the accumulated debris of dead and dying cells. Normal surrounding tissue showed no change. Rh-123 was found to be a potent antitumor drug without causing adverse effects on normal tissue.

Toxicity

Three of five mice died at a dose of 20 mg/kg Rh-123 in DMSO; one of five mice died at 15 mg/kg Rh-123 in DMSO. Two of five mice died that received only 50% DMSO. If the alcohol-glucose solution was used as a diluent, mice tolerated a dose of 20 mg/kg of Rh-123 given every other day for a period of 2 weeks with no mortality.

Rh-123 Cytotoxicity on Cells In Vitro

Figures 2 and 3 are representative of the data obtained in repeat experiments with the different cells studied following exposure to 0–80 μg/ml of Rh-123 for a period of 1–7 days. Figure 2 compares the cytotoxic effects observed with the different cells over a broad (0–80 μg/ml) range of Rh-123 concentrations; Figure 3 shows data from a different experiment covering a narrower range (0–16 μg/ml) of Rh-123 concentrations.

TABLE I. Retention of Rh-123 After 24 Hours in Dye-Free Medium*

Cell line	% Retention of Rh-123
Human prostatic carcinoma	
DU 145	23
LNCaP	
Population 1	24
Population 2	64
PC-3	40
Normal prostate fibroblast	
NPF-209	9

*Relative dye retention was estimated from the shift in mean fluorescence after Rh-123-labeled cells were allowed to recover in dye-free medium for 24 hours. Mean fluorescence values were normalized to 100% at 1 hour labeling with 10 μg/ml Rh-123.

The loss of viable cells was marked in all three types of cancer cells (PC-3, LNCaP, and DU 145) exposed for 2 days or longer to Rh-123 at concentrations as low as 1.25–2 μg/ml; in contrast, viability of NPF diploid cells remained relatively high even after prolonged Rh-123 exposures (Figs. 2, 3). Extent of cytotoxic effects was somewhat different in the three prostate cancer cell lines; PC-3 cells appeared to be the most sensitive to Rh-123 exposure; DU 145 cells appeared to be more refractory than either PC-3 or LNCaP (Figs. 2, 3). There were no significant increases in cytotoxic effects on cells at Rh-123 concentrations higher than 10 μg/ml (Fig. 2). Recovery of Rh-123-treated prostate cancer cells incubated in Rh-123-free, normal growth medium was poor; the proportionate loss in viable cells remained the same or became more acute, suggesting continued loss of viable cells or inhibition of growth, despite restoration to normal growth media (Fig. 2).

Clonogenic assays (Fig. 4) showed that colony growth of PC-3 and LNCaP cells was greatly suppressed following exposure to 10 μg/ml of Rh-123. Growth suppression due to Rh-123 was less marked in DU 145 cells. Complete suppression of colony growth in all three cancer cell lines was noted following Rh-123 10 μg/ml exposure for 72 hours. There was less dose-dependent suppression of normal human adult prostate fibroblasts with maximal suppression (28% of control) following a 72-hour exposure. Total suppression was not achieved even after a 72-hour exposure to 50 μg/ml (data not shown).

The above data correlated well with Rh-123 retention obtained by flow cytometry (Table I). Significant amounts of Rh-123 (73–64%) were retained by the three cancer cell lines, 24 hours after withdrawal of Rh-123. In contrast, over 90% of the Rh-123 taken up by NPF cells was lost within 24 hours following removal of the drug. Thus, these data suggest that the increased toxicity of Rh-123-treated prostate cancer cells observed is due to their selective retention of the drug.

DISCUSSION

Although the destructive effect of Rh-123 on cells in vitro has been reported by several authors [3,12–14] there are few reports of its effect on in vivo solid tumors [15]. Our previously reported studies [4,5], as well as a large series of rats with transplantable tumors treated successfully with Rh-123 (Arcadi, unpublished data), indicate that Rh-123 can destroy transplanted prostate tumors in rats. The studies presented here demonstrated cell destruction by Rh-123 in an autochthonous rat prostate adenocarcinoma with a dose given for a short duration, and also, the preferential sensitivity of three different prostate cancer cell lines.

These studies support the thesis that Rh-123 may be an effective agent for the treatment of metastatic, hormone refractory prostate cancer. Animal studies have demonstrated efficacy and acceptable toxicity.

ACKNOWLEDGMENTS

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EDITORIAL COMMENTS

The study reported by Arcadi and co-workers is a bit of a "good news, bad news" story. The "good news" is that this study addresses a profoundly deficient area—the development of effective chemotherapies for androgen-insensitive prostatic adenocarcinomas and the means to measure their effectiveness rapidly. There is a clear need for the development of assay systems that can monitor, and predict, in vivo sensitivity of tumor cells to therapeutic intervention. While flow cytometric measurements have been proposed for some time [1,2] only recently has the realization that tissue culture systems provide an inadequate model for the study of drug retention and tumor cell killing in vivo [3] begun to have an impact on our thinking. While the use of flow cytometric (or other assays) that can potentially measure tumor cell drug sensitivity looks promising, this area clearly needs well-defined standards and standardized methodologies before it can become clinically useful. The measurement of a certain percentage reduction in the retention of Rh-123 as measured here or any potentially chemotoxic agent is meaningless by itself. These values must be reported in molecules of drug retained per cell (with some understanding of the distribution variation within the population; i.e., is it a 2-fold or 200-fold difference within the tumor?), using standardized methodologies proved useful to predict log kill of tumor cells in vivo. When (and if) we arrive at that point, measurements of drug uptake and retention have a realistic potential to provide clinically useful information on individual cancer patients' tumors.

The bad news is that it involves a comparison of apples and oranges. Here, the connection between rat prostatic adenocarcinoma growing (or not growing) in vivo following Rh-123 (or any drug) treatment, and the toxicity of the same agent on human prostate cancer cell lines growing in vitro is tenuous at best. To demonstrate that Rh-123 suppresses cell growth in tissue culture and to suggest that this supports "the thesis that Rh-123 may be an effective agent for the treatment of metastatic hormone-refractory prostate cancer" (see Discussion) is a connection that has too often failed in the past. As pointed out by Tannock [4] and many others, the local environment of solid malignancies in situ has a profound impact on the responsiveness or nonresponsiveness of cancers where they really count to a patient in his or her body.

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REPLY TO EDITORIAL COMMENTS

We completely agree with the comments. However, we must all remind ourselves that a project must start at some point—our in vitro studies are at a starting, but we believe a significant, phase. We feel our in vivo studies of an autochthonous rat prostate complex adenocarcinoma which show significant cell destruction are unassailable. The commentator, unfortunately, does not comment on the most important feature of Rh-123. It acts by preferentially destroying tumor cell mitochondria with minimal toxicity to normal cells.

—John A. Arcadi, MD